{Exhibit 71}

Fertel R. and Weiss, B., "Measurement of the Activity of Cyclic Nucleotide Phosphodiesterases with Firefly Luciferin-Luciferase Coupled Assay Systems," Methods in Enzymol. Vol LVII, Bioluminescence and Chemiluminescnece, DeLuca, M.A. (Ed.) 94-96 (1978)

extremely heat-stable protein, back production of ATP will occur (2 ADP $\overset{MK}{\rightleftharpoons}$ AMP + ATP) following the heat deactivation of coupled Reactions (3) and (4). By maintaining relatively low concentrations of ATP [i.e., ADP after Reaction (1)] and of GK, the effects of MK contamination are eliminated. If sufficiently high levels of ADP are present within the GTP extracts (>200 ng of ADP ml^{-1}), a small amount of light will be produced, after sample injection, owing to MK activity contained within the crude luciferase preparations. The amount of light emitted is less than 1% of the activity resulting from an equimolar concentration of GTP; however, if necessary, this source of interference can be evaluated (and corrected for) by measuring the sum of the concentrations of ATP and ADP within each sample extract[20] and relating these values to the reactivity of standard ADP solutions.

Acknowledgments

The author expresses his appreciation to Dr. O. Holm-Hansen for comments, criticism, and encouragement offered during the course of this research and the preparation of this chapter. Dr. F. Azam and Ms. L. Campbell critically reviewed the original manuscript and offered helpful suggestions for improvement. The methodology described in this report was developed under ERDA contract EY-76-C-03-0010 P.A. 20.

[10] Measurement of the Activity of Cyclic Nucleotide Phosphodiesterases with Firefly Luciferin–Luciferase Coupled Assay Systems

By RICHARD FERTEL and BENJAMIN WEISS

The procedures described below are designed to measure cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase (EC 3.1.4.17) by reaction sequences using firefly luciferin and luciferase in the final step. These assay systems are simple, sensitive, inexpensive, and reproducible.

METHODS IN ENZYMOLOGY, VOL. LVII

ISBN 0-12-181957-4

Intracellular concentrations of adenosine 3',5'-cyclic monophosphate (cyclic AMP) and guanosine 3',5'-cyclic monophosphate (cyclic GMP) affect a number of biochemical and physiologic processes in the cell.[1-6] Accordingly, certain cell functions may be controlled by altering the cyclic nucleotide concentrations in that cell. One way to influence the concentration of the cyclic nucleotides is by activating or inhibiting cyclic nucleotide phosphodiesterases, which catalyze the hydrolysis of the cyclic nucleotides to their 5'-monophosphate analogs. For this reason, these enzymes have been the subject of a number of investigations,[6] and a variety of methods have been devised to measure their activity.[7-10] We have developed for these enzymes assay procedures based on the quantitative coupling of the product of the phosphodiesterase reaction [either adenosine 5'-monophosphate (5'-AMP) or guanosine 5'-monophosphate (5'-GMP)] to adenosine-5'-triphosphate [ATP].[11,12] The concentration of ATP in the reaction mixture is then determined by means of the firefly luciferin–luciferase reaction.[13]

Principle of the Assay Systems

Cyclic-AMP Phosphodiesterase

In this reaction sequence, the phosphodiesterase converts cyclic AMP to its degradation product, 5'-AMP. The 5'-AMP, in the presence of a very low concentration of ATP, which serves as a phosphate donor, is converted by the enzyme myokinase (EC 2.7.4.3) to adenosine-5'-diphosphate

[1] G. A. Robison, G. G. Nahas, and L. Triner, eds. *Ann. N. Y. Acad. Sci.* **185** (1971).
[2] P. Greengard, R. Paoletti, and G. A. Robison, eds. "Advances in Cyclic Nucleotide Research," Vol. 1, Raven, New York, 1972.
[3] P. Greengard and E. Costa, eds. *Adv. Biochem. Psychopharmacol.* **3** (1970).
[4] N. D. Goldberg, R. F. O'Dea, and M. K. Haddox, *in* "Advances in Cyclic Nucleotide Research" (P. Greengard and G. A. Robison, eds.), Vol. 2, p. 155. Raven, New York, 1973.
[5] B. Weiss, ed. "Cyclic Nucleotides in Disease." Univ. Park Press, Baltimore, Maryland, 1975.
[6] B. Weiss, and R. Fertel, *Adv. Pharmacol. Chemother.* **14**, 189 (1977).
[7] R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.* **237**, 1244 (1962).
[8] W. J. Thompson and M. M. Appleman, *J. Biochem.* **10**, 311 (1971).
[9] C. R. Filburn and J. Karn, *Anal. Biochem.* **52**, 505 (1973).
[10] W. Y. Cheung, *Anal. Biochem.* **28**, 182 (1969).
[11] B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* **45**, 222 (1972).
[12] R. Fertel and B. Weiss, *Anal. Biochem.* **59**, 386 (1974).
[13] B. L. Strehler and J. R. Totter, *Arch. Biochem. Biophys.* **40**, 28 (1952).

(ADP). ADP is then converted to ATP in the presence of phosphoenolpyruvate, which serves as a phosphate donor, and the enzyme pyruvate kinase (EC 2.7.1.40), which catalyzes the reaction.

Under the conditions specified below, the 5′-AMP is rapidly and completely converted to ATP, which is then measured using the firefly luciferin–luciferase reaction. The entire sequence is shown below:

$$\text{Cyclic AMP} \xrightarrow{\text{phosphodiesterase}} \text{5′-AMP}$$

$$\text{5′-AMP} + \text{ATP} \overset{\text{myokinase}}{\rightleftharpoons} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{phosphoenolpyruvate} \overset{\text{pyruvate kinase}}{\rightleftharpoons} 2\ \text{ATP} + 2\ \text{pyruvate}$$

$$\text{ATP} + \text{luciferin} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{AMP} + \text{PP}_i + \text{oxyluciferin} + \text{CO}_2 + \text{light}$$

Cyclic-GMP Phosphodiesterase

In this reaction sequence, the 5′-GMP formed as the product of the phosphodiesterase reaction alters the equilibrium of the reaction catalyzed by guanosine-5′-monophosphate kinase (EC 2.7.4.8). This leads to a decrease in ATP that is proportional to the concentration of 5′-GMP formed. The ATP concentration is then determined by reaction with firefly luciferin and luciferase. The reaction sequence is as follows:

$$\text{Cyclic GMP} \xrightarrow{\text{phosphodiesterase}} \text{5′-GMP}$$

$$\text{5′-GMP} + \text{ATP} \overset{\text{guanosine-5′-monophosphate kinase}}{\rightleftharpoons} \text{GDP} + \text{ADP}$$

$$\text{ATP} + \text{luciferin} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{AMP} + \text{PP}_i + \text{oxyluciferin} + \text{CO}_2 + \text{light}$$

Reagents Used in the Assay Systems

Reagents for the Cyclic-AMP Phosphodiesterase Assay

Reagent A

Glycylglycine buffer, 150 m*M* pH 8.0
Ammonium acetate, 75 m*M*
Magnesium chloride, 9 m*M*